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Test and Evaluation of Field-Deployable Infectious Disease Diagnostic Assays in Support of the Joint Biological Agent Identification and Diagnosis System (JBAIDS): Malaria (Plasmodium/JBAIDS)

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

None.

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Air Force Medical Support Agency (AFMSA), Research, Development and Innovations Directorate (SG9), Office of the Surgeon General (AF/SGR) Falls Church, Virginia

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None.

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Approved for Public Release; Distribution Unlimited.

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This project was conducted under MOA between Walter Reed Army Institute of Research (WRAIR), Silver Spring, Maryland & 59th Medical Wing (MDW) Lackland AFB, Texas (MOA 2007 - 2012, Agreement No.: DODI 4000.19; AFI 25-201).

14. ABSTRACT

A Plasmodium genus dual-fluorogenic, hydrolysis probe (TaqMan) polymerase chain reaction (PCR) assay was developed using real-time PCR instrumentation (MiniOpticon Real-Time PCR System, Bio-Rad, Hercules, CA). The Plasmodium genus assay is sensitive, specific, and provides rapid detection from infected mosquitoes and malaria patient blood. Operational applications are FHP disease surveillance and ultimately molecular-based diagnostics. A unique 18S rRNA gene sequence conserved in Plasmodium species was targeted for the design of oligonucleotide primers and probe. The LoD of the Plasmodium genus TaqMan assay was established at 10 genome equivalents using P. vivax plasmid and P. falciparum plasmid. The assay was 100% (17/17) concordant in testing using a diverse panel of Plasmodium species and strains prepared as plasmid control DNA. The assay was 100% (5/5) sensitive and 100% (13/13) specific in testing with a diverse panel of human malaria Plasmodium species and non-malaria pathogens. No cross-reaction occurred with human blood samples. Transfer to the RAPID/JBAIDS presents no technical challenges. Assay [illegible]

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a. REPORT	b. ABSTRACT	c. THIS PAGE			James C. McAvin
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Clinical Research Division
59th Medical Wing, Lackland AFB, Texas

And

Armed Forces Research Institute for the Medical Sciences (AFRIMS),
Bangkok, Thailand

Final Report

AFMSA O&M FY10 Project (FWH20090194E):

Test and Evaluation of Field-Deployable Infectious Disease Diagnostic Assays in Support of the Joint Biological Agent Identification and Diagnosis System (JBAIDS):

Malaria (*Plasmodium*/JBAIDS)

May 31, 2012

Reporting Period: October 1, 2010 to May 31, 2012

Principal Investigator: Col James A. Swaby
59th Medical Wing, Lackland, AFB Texas

Report Prepared by:

James C. McAvin (Designated PI July 2011)
Clinical Research Division/59th Medical Wing

And

Ratree Takhampunya, Ph.D. (Co-I),
Department of Entomology/AFRIMS

Project Objectives

1. Provide scholarly and challenging Graduate Medical Education opportunities.

   Graduate Medical Education (GME) was not completed during this O&M FY10 project.

   Note: under AFMSA FY12 - FY13 RDT&E funded projects a formal GME training program has been established by 59th MDW and AFRIMS investigators.

2. Complete RAPID-based test and evaluation data to support AFPMB approval of the RAPID-based *Plasmodium f. and v.* assays for use in vector/vector-borne disease surveillance using the RAPID.

   The primary objective of this project was accomplished - development of FHP malaria causative agent surveillance capability.

   The development of these assays has been a challenge to 59th MDW and WRAIR/AFRIMS investigators. This work was originally undertaken by WRAIR during 2006. Joint efforts under MOA (FY07 - FY09) were unsuccessful. This O&M FY10 project was not executed as planned primarily due to political unrest which resulted in withdrawal of CRD/59th MDW technical support and equipment.

   During MAR - MAY 2012 AFRIMS collaborators provided PI TDY funding, technical support, facility use, and supplies allowing the development of a *Plasmodium* genus TaqMan assay.

3. Complete JBAIDS-based test and evaluation data to support JBAIDS program manager approval of the JBAIDS-based *Plasmodium f. and v.* assays for use in environmental (non-human) surveillance using the JBAIDS.

   The CRD/59th MDW provided technical support and RAPID/JBAIDS were withdrawn prior to completion of project activities.

4. Qualified by the JBAIDS program manager as a candidate assay for future research targeted at getting FDA-clearance for human diagnostics on the JBAIDS under a separate research proposal.

   Validation testing using the RAPID and JBAIDS must be completed prior to submissions to the AFPMB and JPO/JBAIDS project manager.

Summary

During MAR through MAY 2012 the fundamental objective of this AFMSA FY10 O&M study was accomplished; real-time malaria causative agent detection capability. A highly sensitive and specific *Plasmodium genus* TaqMan PCR wet assay was developed using real-time PCR instrumentation (MiniOpticon Real-Time PCR System, Bio-Rad, Hercules, CA). This assay is applicable for use with diverse real-time PCR instrumentation to include RAPID and JBAIDS.

Develop of *Plasmodium* real-time PCR detection capability has been challenging. During 2006, the Division of Entomology, WRAIR designed and developed a *Plasmodium* genus TaqMan assay (16S rRNA gene target). The chemistry applied was minor groove binder (MGB). During 2007, the assay was redesigned and reformulated using standard TaqMan chemistry to meet JPO/JBAIDS requirements. During FY07 - FY08 *Plasmodium* genus assay optimization, freeze-dried reagent production, and preliminary sensitivity and specificity testing were successfully completed. However, during field evaluation the assay failed to meet acceptance test criteria due to probable thermal-stability issues. Testing conducted during FY09 showed cross-reactivity. Resolution of cross-reactivity (potential thermal stability issues) was not resolved by the completion of the study. Under the AF/SGR AFMSA O&M FY10 'Plasmodium Project', existing *Plasmodium* genus, *P. falciparum*, and *P. vivax* TaqMan assays were proposed for transfer to the RAPID/JBAIDS. Project activities were not executed as planned primarily due to political unrest. Malaria causative agent detection capability was accomplished during March through May 2012. A highly sensitive and specific *Plasmodium* genus TaqMan PCR assay was developed and successfully tested.

Project funding was provided by the Air Force Medical Support Agency (AFMSA), Research, Development and Innovations Directorate (SG9), Office of the Surgeon General (AF/SGR) Falls Church, Virginia and the Military Infectious Diseases Research Program (MIDRP), USAMRC, Fort Detrick, Frederick, Maryland. Project execution was conducted by the Armed Forces Research Institute for the Medical Sciences (AFRIMS) and Clinical Research Division (CRD)/59th MDW. This project was jointly funded and executed under memorandum of agreement (MOA) between Walter Reed Army Institute of Research (WRAIR), Silver Spring, Maryland & 59th Medical Wing (MDW) Lackland AFB, Texas (MOA 2007 - 2012. Agreement No.: DODI 4000.19; AFI 25-201).

AFMSA O&M FY10 'Plasmodium Project' funding level was $146K. Distributions were $92K to the 59th CRD and $54K to WRAIR/AFRIMS. Results from 2006 - 20012 joint efforts are described in this report.

Products Completed

A *Plasmodium genus* dual-fluorogenic, hydrolysis probe (TaqMan) polymerase chain reaction (PCR) assay was developed using real-time PCR instrumentation (MiniOpticon Real-Time PCR System, Bio-Rad, Hercules, CA). The *Plasmodium genus* assay is sensitive, specific, and provides rapid detection from infected mosquitoes and malaria patient blood. Operational applications are FHP disease surveillance and ultimately molecular-based diagnostics. A unique

18S rRNA gene sequence conserved in *Plasmodium* species was targeted for the design of oligonucleotide primers and probe.

The LoD of the *Plasmodium* genus TaqMan assay was established at 10 genome equivalents using *P. vivax* plasmid and *P. falciparum* plasmid. The assay was 100% (17/17) concordant in testing using a diverse panel of *Plasmodium* species and strains prepared as plasmid control DNA. The assay was 100% (5/5) sensitive and 100% (13/13) specific in testing with a diverse panel of human malaria *Plasmodium* species and non-malaria pathogens. No cross-reaction occurred with human blood samples. Transfer to the RAPID/JBAIDS presents no technical challenges.

Assay chemistry and formulation allows use on diverse real-time PCR analytic platforms. The *Plasmodium genus* assay is a likely candidate for transfer to the DoD NGDS.

Use by the Government

This study addresses DoD priorities in protecting the health of soldiers. Malaria is ranked first among the top 40 diseases in the DoD global risk-severity index and recognized as a military significant disease.

With no licensed vaccine available, accurate diagnostics and real-time disease surveillance capability are essential in efficacious treatment and mitigating outbreaks. The assays facilitate timely assessments of transmission risk and time-critical implementation of personal protection and focused transmission control measures. The assay will be used in US Military mosquito-borne disease agent surveillance programs.

Assay primer and probe sequences will be classified as Government Furnished Material (GFM) and Government Furnished Information (GFI) respectively and serve as deliverable to the Joint Program (JPEO) for integration in Critical Reagent Program (CRP) and potential Federal Drug Administration (FDA) clearance using the DoD Joint Biological Agent Identification and Diagnostic System (JBAIDS). The oligonucleotides are candidates for transfer to the DoD "Next Generation Diagnostic System". The ultimate objective of the DoD is commercialization of the assay for disease surveillance and human diagnostic uses.

Commercial Application

Malaria is one of the most widespread mosquito-borne diseases in the world. Commercializing the assay will allow public health organizations globally to enhance malaria surveillance programs and potentially provide an aid in diagnosis.

Purpose

To test and evaluate critical Force Health Protection disease surveillance assays in support of the JBAIDS program. Assays evaluated included mosquito-borne causative agents for malaria. This work was undertaken to address the critical need for field expedient assays to evaluate the complete epidemiology of these diseases to support "real-time" preventative and control

measures. The ability of medical personnel to accurately survey and recognize infectious disease threats in an operational environment is a high priority. The rapid identification of an infectious agent will allow for prompt, appropriate treatment, thereby minimizing morbidity and mortality. Additionally, knowledge about a specific infectious disease threat will allow for the implementation of prevention and control efforts to protect the fighting force.

Problem

Naturally occurring infectious diseases pose a significant threat to military operations and can account for up to 80% of all casualties. Over 25% of marines deployed to Liberia in 2003 were infected with the potentially fatal falciparum malaria. Between 2003 and 2005, over 2,000 military personnel deployed to Iraq were infected with leishmaniasis. A variety of other infectious diseases have impacted every military operation conducted in the past century.

The Joint Biological Agent Identification and Diagnosis System (JBAIDS) is an $80 million acquisition program that is currently fielding approximately 425 JBAIDS platforms. Idaho Technology, Inc. (ITI) is the prime contractor for the JBAIDS program, responsible for delivering both the platform and associated assays. The JBAIDS program is currently funding the development of the 10 "first tier" assays contained in the JBAIDS Operational Requirements Document (ORD). All 10 of these agents are Biological Warfare Agents. The JBAIDS ORD also requires the development of an additional 10 "second tier" assays for naturally-occurring infectious disease agents. Funding has not been provided for the development, testing and evaluation of these "second tier" assays.

Background

This project was conducted through WRAIR & 59th MDW MOA FY07 - FY12. During 2006, the Division of Entomology, WRAIR designed and developed a *Plasmodium* genus TaqMan assay (16S rRNA gene target). The chemistry applied was minor groove binder (MGB). TaqMan MGB probes are typically used for allelic discrimination assays to provide for more accurate allelic discrimination. The WRAIR used MGB chemistry because it also provides more flexibility in probe design. This chemistry increases the melting temperature (Tm) thus allowing the design of shorter probes where target sequence is limited such as the *Plasmodium* genus 16S rRNA target sequences. The results of the assay were promising (Fig. 1).

However, JBAIDS TaqMan chemistry requirements prohibit the use of MGB therefore the assay was re-designed using conventional TaqMan chemistry, a probe with TAMRA dye as the quencher dye. The assay was optimized using wet JBAIDS reagents, LoD established, and preliminary specificity testing conducted. Wet assay formulation was used to prepare freeze-dried (FD) assays (Idaho Technology, Inc.). The FD assay was transitioned to the RAPID/JBAIDS and platforms and the performance compared to an existing TaqMan format (Cepheid SmartCycler). Laboratory testing using the RAPID/JBAIDS was conducted at the Entomology Department, AFRIMS and field validation testing subsequently conducted at AFRIMS Malaria field site at Mae Sot.

During FY07 - FY08 *Plasmodium* genus assay optimization, FD reagent production, and preliminary sensitivity and specificity testing were successfully completed (Fig. 2-4). However, under field test conditions the assay failed to meet acceptance test criteria presumably due to probable thermal-stability issues (results are shown in figures 5-7). Testing conducted during FY09 showed cross-reactivity that remained unresolved at the completion of the study (representative results are shown in figures 7).

During the FY07 – FY09 studies, *Plasmodium* genus assay NALOD was established with wet reagents on the RAPID, and the preliminary lower limit of detection for this assay was found to be roughly 7.5 fg/ul using *Plasmodium falciparum* template strain 3D7G1. This concentration of Plasmodium DNA corresponds to approximately 3 genome copies of *Plasmodium falciparum* DNA. Freeze-dried reagents for the *Plasmodium* genus assay were prepared by ITI using the optimized conditions of the wet reagents, and the NALOD was confirmed on the JBAIDS instrument using the standard JBAIDS thermocycling conditions for DNA target. The assessment performed by ITI showed robust amplification of the genomic DNA target (Appendix A).

Plasmodium genus FD assays were hand-carried to the Entomology Department, AFRIMS to support laboratory validation testing and field evaluation at the AFRIMS Malaria field site at Mae Sot, Thailand. Just prior to field testing, an abbreviated validation study of the freeze-dried assays confirmed LoD, sensitivity and specificity performance (Fig. 5).

Figure 1. Amplification plots of *Plasmodium* genus 16S RNA target using MGB chemistry.

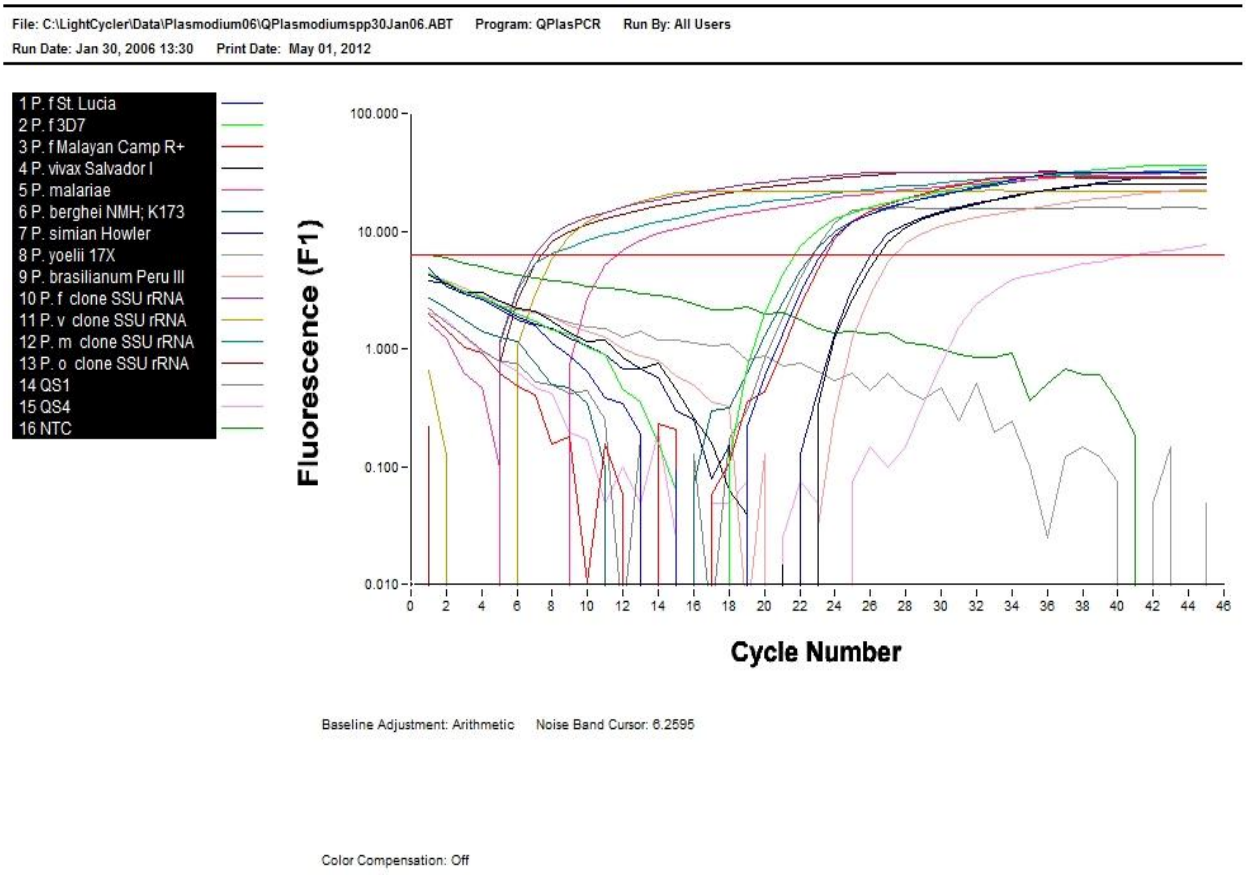


Figure 2. Amplification plots of *Plasmodium* genus MgCl2 optimization using TaqMan chemistry.

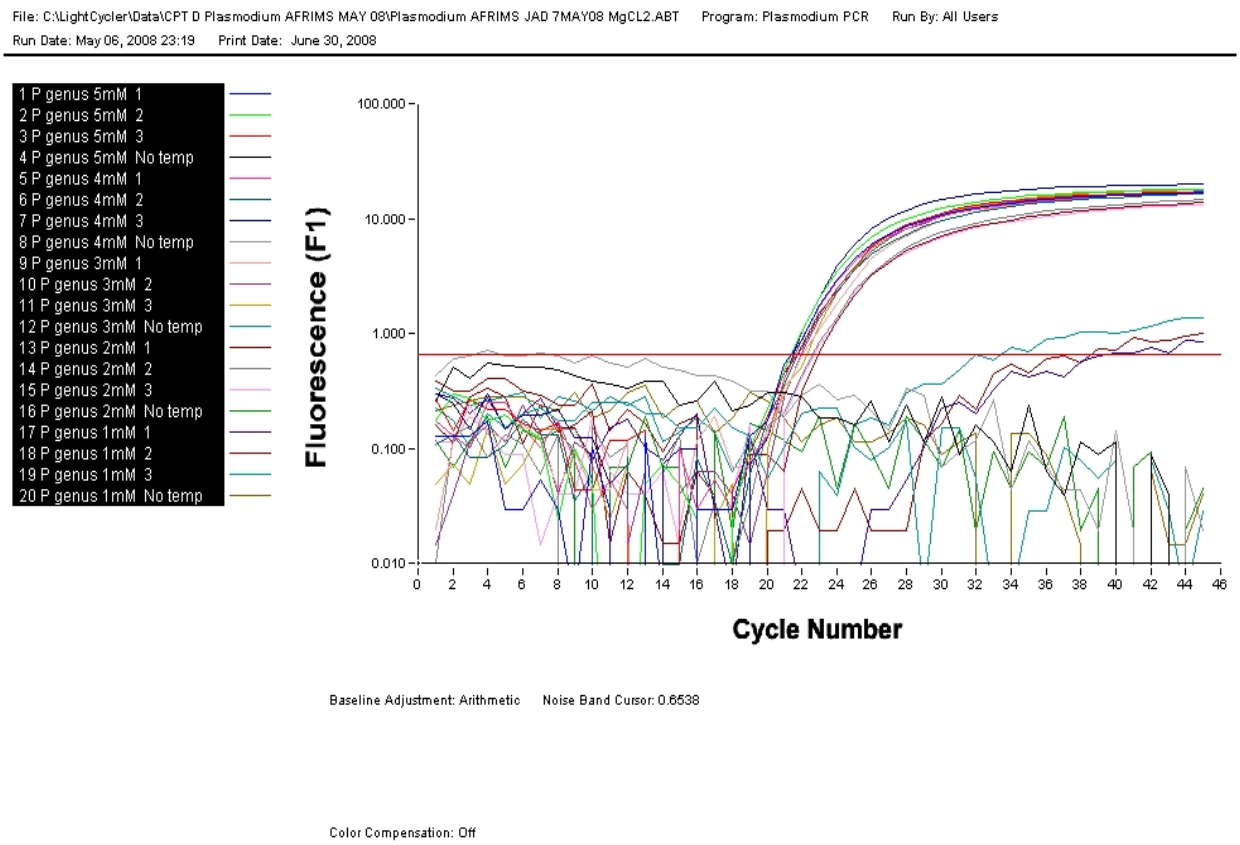


Figure 3. Amplification plots of *Plasmodium* genus primer titration using TaqMan chemistry.

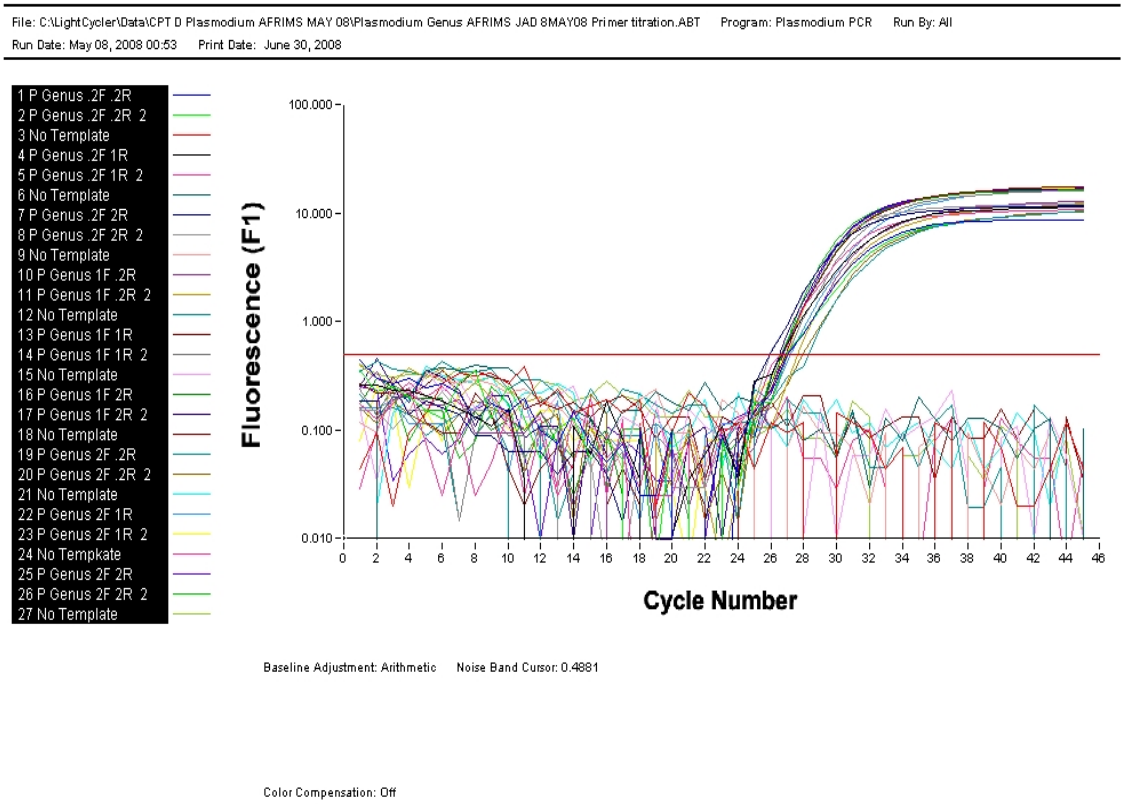


Figure 4. Amplification plots of *Plasmodium* genus TaqMan probe titration.

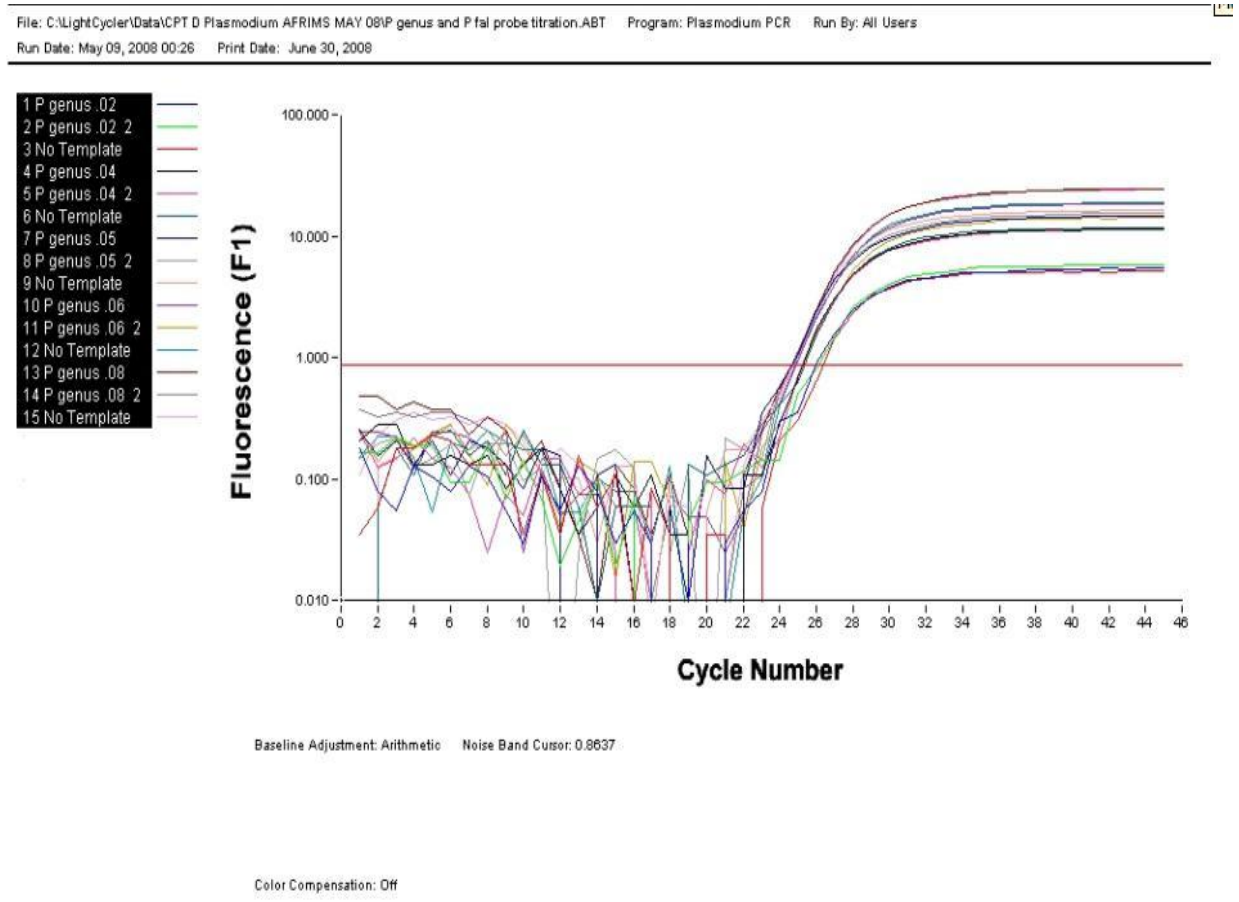
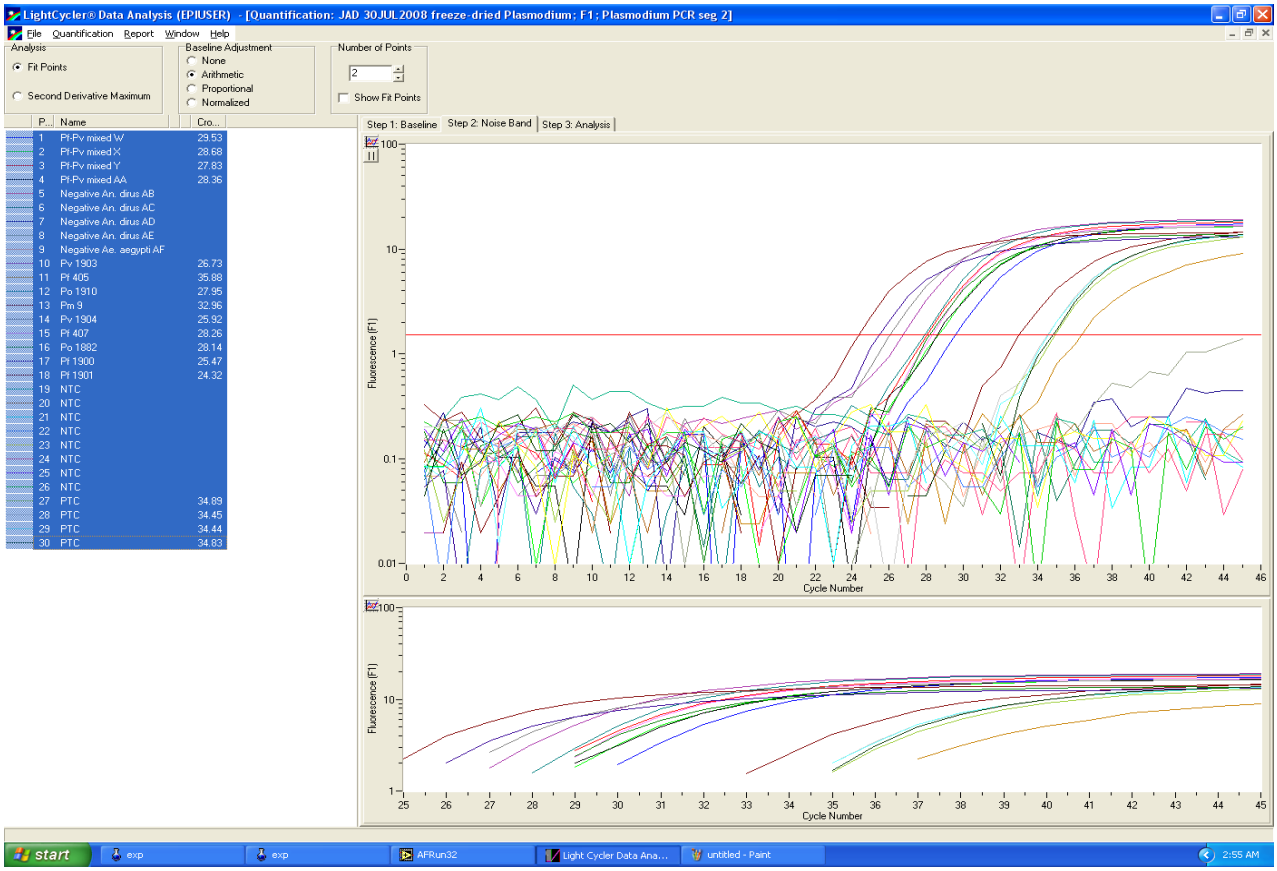


Figure 5. *Plasmodium* genus TaqMan RAPID assay sensitivity and specificity test results.



This validation work was followed by vector surveillance studies at the Mae Sot field site in Thailand during the week of 14 July, 2008. The PI, co-investigators, and GME student traveled to the Mae Sot province for initial field-based testing. Collections and processing of genomic DNA from female anopheles mosquitoes proceeded the testing of the assay on the JBAIDS and RAPID. Once the first set of mosquitoes was collected and processed, an initial reagent QC run was performed. Immediately, the presence of false-positives was discovered (Fig. 6). The false positives initially were attributed to the presence of contamination, so the entire PCR working section of the field laboratory was decontaminated, and the samples were re-run. Again, false positives were noted. The following run (performed at the same location) was a complete rotor run of negative controls and positive controls (28 negatives and 4 positives). 30% of the negative controls produced false positives.

The work was subsequently moved near the anopheles collection site at Mae Sot, and the process of running samples of extracted anopheles gDNA resumed; however, false-positives were observed (Fig. 7 - 8). Additional rotor runs of negative controls were run; however, approximately 30% of the negative controls were again coming up positive. At this point, we began to consider the possibility that there may be a problem with the thermostability of the freeze-dried chemistry or specific lots of the reconstitution buffer. The following day (Tuesday, July 17, 2008), we tested all available lots of the reconstitution buffer in an attempt to rule out

the possibility of a problem with a specific lot of buffer. The results from this experiment indicated no link of the false-positive problem to a particular lot of reconstitution buffer. The team returned to AFRIMS (Entomology Division) on Friday, July 18, 2008, and conducted repeat testing using a specific portion of freeze-dried reagents that were maintained at 4 degrees Celsius for the duration of the TDY. Results from these experiments deviated from the false-negative results we had encountered in previous experiments with freeze-dried reagents maintained at ambient temperatures.

Laboratory versus field test results indicated probe degradation when stored at ambient temperatures. Examples of assay performance when tested under cold chain and ambient temperatures are shown below (Fig. 9 and 10). The probable cause of probe degradation was assay thermal instability resulting from an uncoupling of probe re-design with reformulation either at the research or production level.

Figure 6. *Plasmodium* genus RAPID TaqMan assay QC field 15 Jul test results.

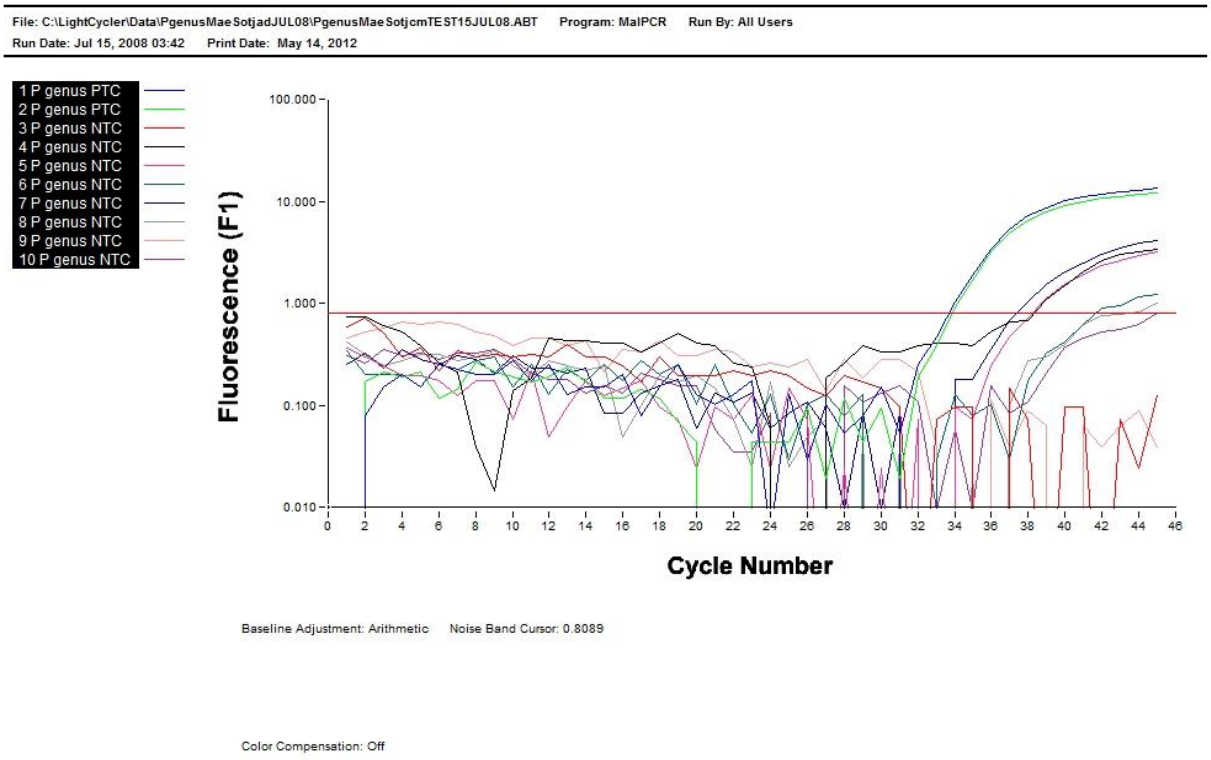


Figure 7. *Plasmodium* genus TaqMan RAPID assay field test 17 JUL results.

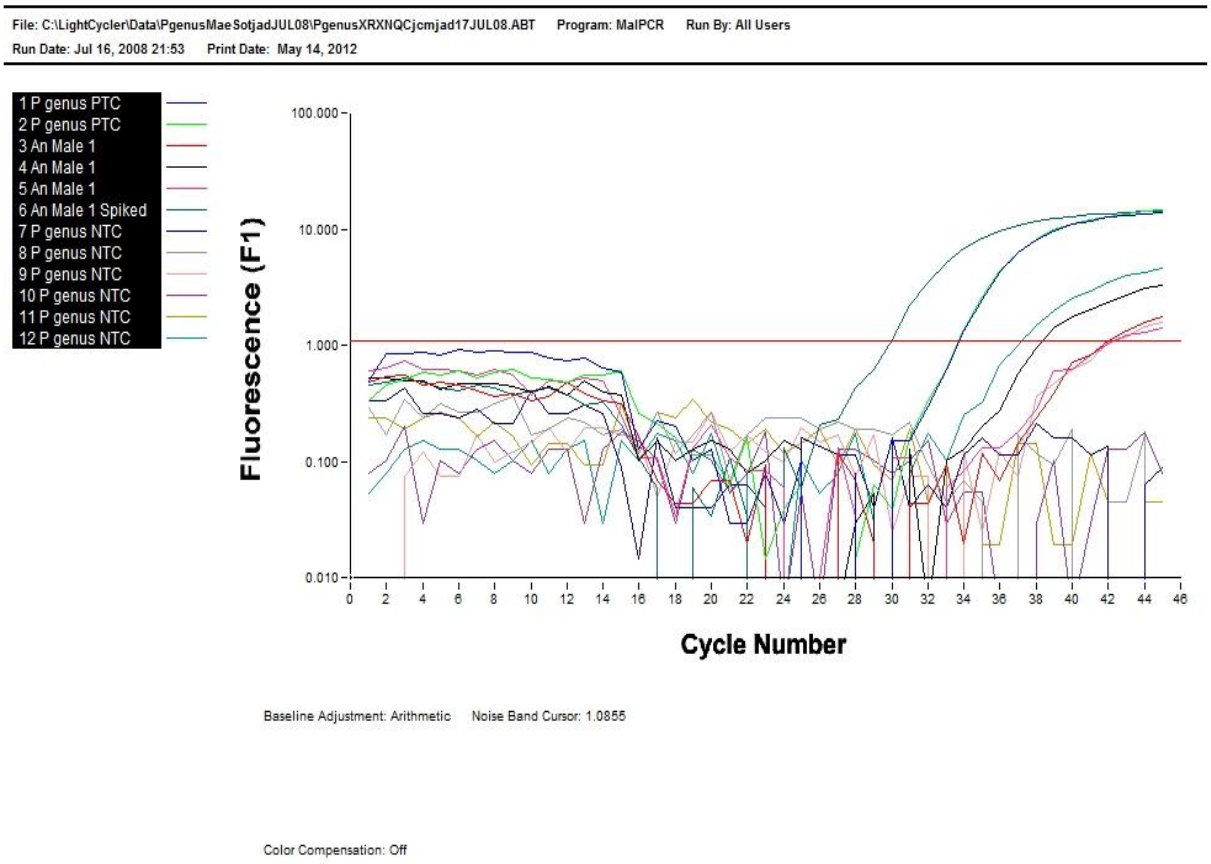


Figure 8. *Plasmodium* genus TaqMan assay field 17 JUL test results.

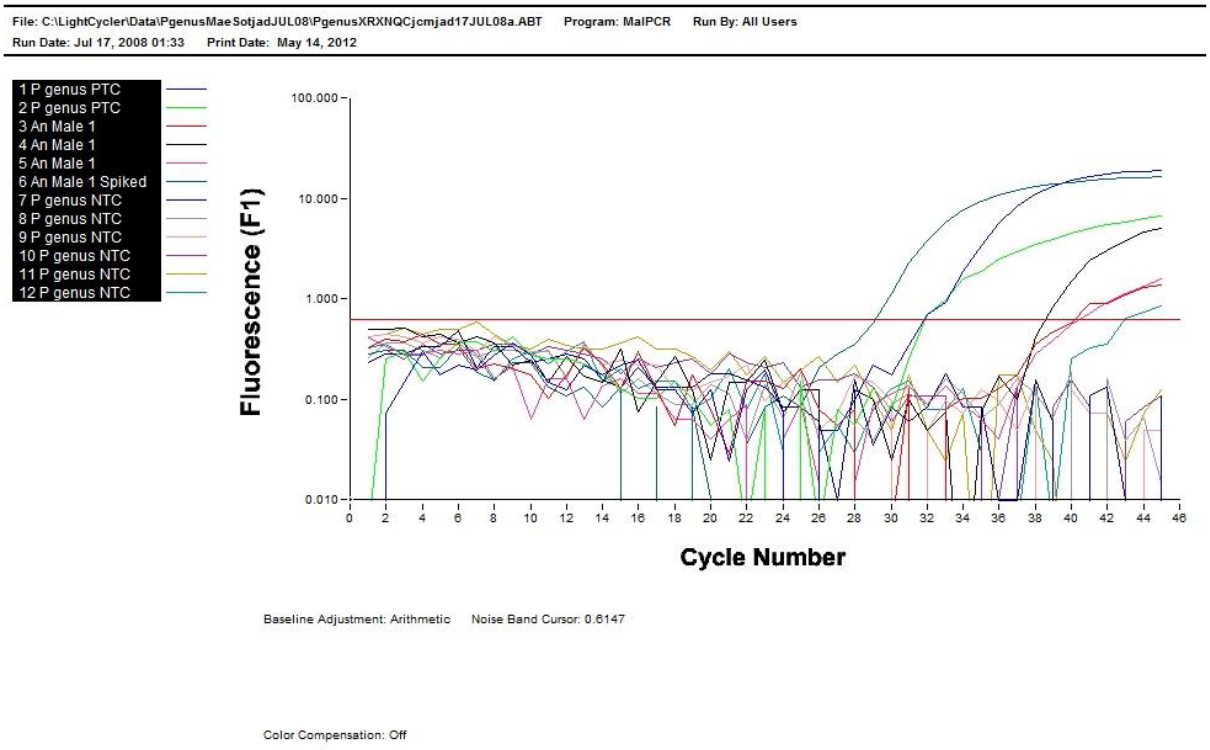


Figure 9. Post field test evaluation: performance of the *Plasmodium* genus TaqMan RAPID assay when stored at 4º centigrade

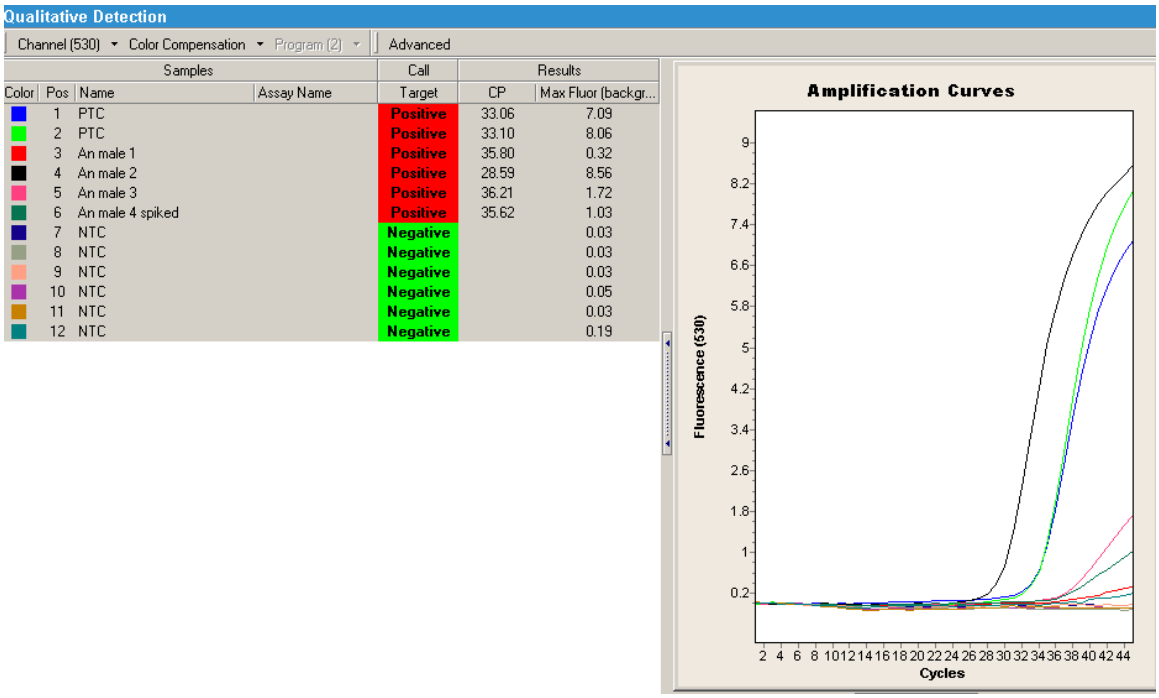
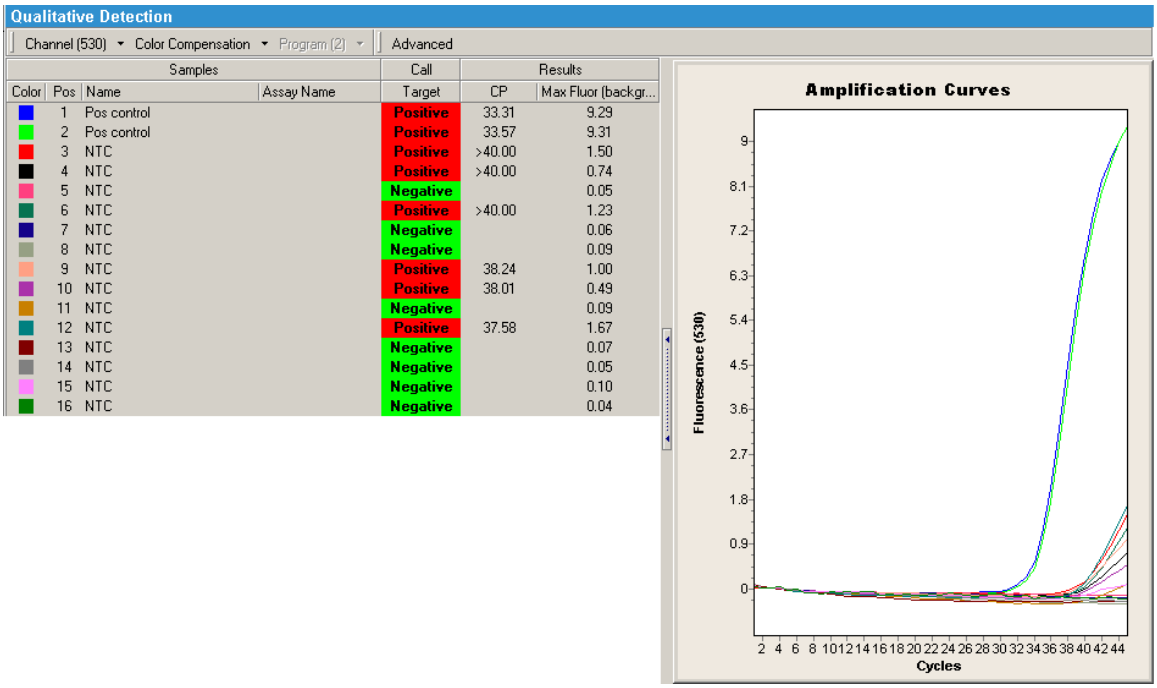


Figure 10. Post field test evaluation: performance of the *Plasmodium* genus TaqMan RAPID assay when exposed to ambient temperatures



Coordination on the resolution of potential thermal stability issues, production of assays, and planning for re-testing was attempted during FY09. The intent was to thermal-stabilize the assay and establish AFPMB and JBAIDS program manager approved RAPID/JBAIDS malaria causative agent detection capability. However, during the FY09 study FD assay QC testing conducted at WRAIR cross-reactivity was observed with uninfected mosquito homogenate and NTC. As such, primer and probe sequences required redesign.

Under the AF/SGR AFMSA O&M FY10 *Plasmodium* project, WRAIR developed *Plasmodium* genus, *P. falciparum*, and *P. vivax* TaqMan assays were proposed for transfer to the RAPID/JBAIDS. Project activities were not executed as planned.

During March through May, 2012 AFRIMS collaborators provided funding, technical staff, facility use, and supplies to complete AFMSA O&M FY10 Plasmodium Project funded deliverables. The core of the project was successfully completed, malaria causative agent detection capability. A *Plasmodium* genus TaqMan PCR assay was developed and successfully tested. Results are shown below.

Results

Primers and Probes *Plasmodium* genus primers and TaqMan probe sequences were designed by the Department of Entomology, AFRIMS. The target is a 18S rRNA gene sequences conserved across *Plasmodium* species.

Forward Primer 5'-TATTCAGATGTCAGAGGTGAAATTC-3' sense

Reverse Primer 5'-ATGAGAAATCAAAGTCTTTGGGTTC-3' antisense

GenusProbe 5'FAM-ACGATCAGATACCGTCGTAATCTT-TAMRA3' sense

Platinum Quantitative PCR supermix-UDG with ROX kit (Invitrogen, USA)

Component	ul/reaction	Final concentration
DDW	9.7	-
2xPlatinum qPCR SuerMix-UDG	12.5	1X
20pmole/ul forward primer	0.4	8 pmoles
20pmole/ul reverse primer	0.4	8 pmoles
10 pmole/ul probe genus	0.5	200 nM
50 mM MgCl2	0.5	4 mM
DNA	1	
Total	25	

Cycle Condition: Bio-Rad real-time PCR

50.0 C for 2 min (UDG incubation)

95.0 C for 2 min

40 cycles of

95.0 C for 15 sec
60.0 C for 30 sec

Primer and probe sequences and TaqMan assay formulation as well as cycling conditions are appropriate for RAPID/JBAIDS instrumentation. Assay transfer to the RAPID/JBAIDS requires demonstration that LoD test results are reproducible. Sensitivity and specificity testing requires a bridging study to confirm assay performance.

Result of BLAST Searches of Amplicon Product

Amplicon = 254 bp

5’ tattcagatgtcagaggtgaaattcttagattttctggagacaaacaactgcgaaagcatttgcctaaaatacttccattaatcaagaacgaaagttaagggagtgaagacgatcagataccgt
Cgtaatcttaaccataaactatgccaactaggttttggatgaaaaatttttaaataagagaattcctttcggggaaatttcttagattgcttctttcagtaccttatgagaaatcaaagtctttgggttc 3’

Nucleotide Sequence

Molecule type: nucleic acid

Query Length : 254

Description: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences)

Program: BLASTN 2.2.26+ Citation

Reference : Stephen F. Altschul, Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Search parameter name	**Search parameter value**
Program	blastn
Word size	7
Expect value	1000
Hitlist size	100
Match/Mismatch scores	1,-3
Gapcosts	5,2
Filter string	F

Genetic Code 1

Database

Database parameter name	Database parameter value
Posted date	May 21, 2012 2:13 PM
Number of letters	41,121,908,494
Number of sequences	16,052,176
Entrez query	none

Karlin-Altschul statistics

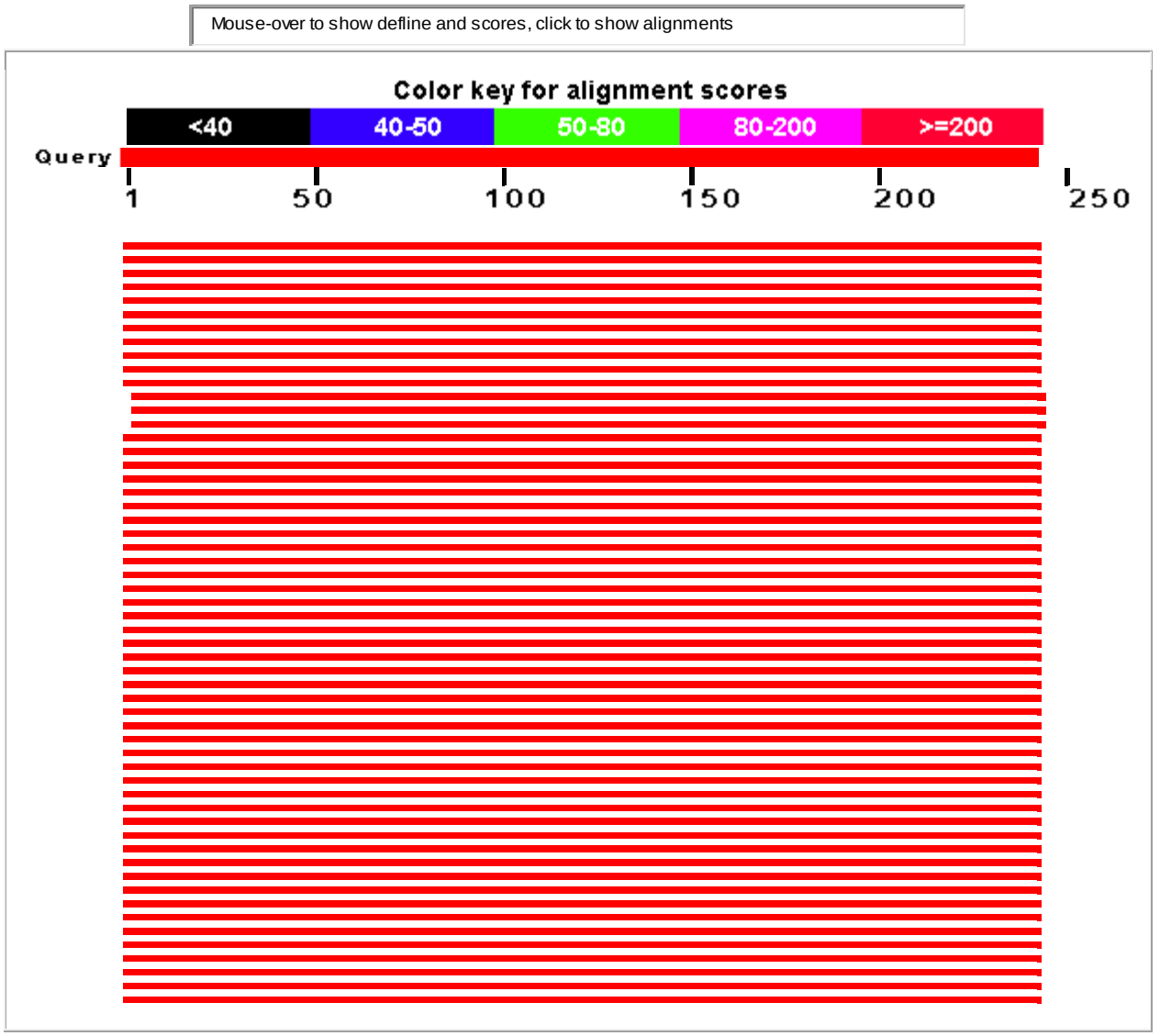
Params	Ungapped	Gapped
Lambda	1.37406	1.37406
K	0.710603	0.710603
H	1.30725	1.30725

Results Statistics

Results Statistics parameter name	Results Statistics parameter value
Length adjustment	22
Effective length of query	232
Effective length of database	40768760622
Effective search space	9458352464304
Effective search space used	9458352464304

Graphic Summary

Distribution of 100 Blast Hits on the Query Sequence



Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HQ697267.1	Plasmodium ovale isolate SM10-210 18S ribosomal RNA gene, partial sequence	504	504	100%	2e-139	100%
HQ697266.1	Plasmodium ovale isolate SM04-119 18S ribosomal RNA gene, partial sequence	480	480	100%	3e-132	99%
AB182490.1	Plasmodium ovale gene for small subunit ribosomal RNA, compolete sequence, classic type 2	480	480	100%	3e-132	99%
DQ104413.1	Plasmodium ovale small subunit ribosomal RNA gene, partial sequence	472	472	100%	6e-130	98%
AB182489.1	Plasmodium ovale gene for small subunit ribosomal RNA, complete sequence, classic type 1	472	472	100%	6e-130	98%
AF145337.1	Plasmodium ovale small subunit ribosomal RNA gene, partial sequence	472	472	100%	6e-130	98%
L48987.1	Plasmodium ovale small subunit ribosomal RNA gene, complete sequence	472	472	100%	6e-130	98%
L48986.1	Plasmodium ovale clone 26 SSU ribosomal RNA (SSUrRNA) gene, complete sequence	472	472	100%	6e-130	98%
DQ845247.1	Plasmodium ovale isolate QPID_ovale18s_06-01 18S ribosomal RNA gene, partial sequence	442	442	100%	6e-121	97%
AJ001527.1	Plasmodium ovale small subunit ribosomal RNA gene	442	442	100%	6e-121	97%
X99790.1	P.ovale rRNA gene	442	442	100%	6e-121	97%

Only top ten sequences producing significant alignments shown.

***Plasmodium* genus TaqMan Assay Optimization**

Representative results of assay optimization testing are shown below (Figure 1). Optimization was conducted by varying $MgCl_2$ concentrations, 3 mM, 4 mM, 5 mM, 6 mM using constant concentrations of primer at 8 pM and probe at 200nM (Table 1). Optimal cofactor concentration was established at 4 mM $MgCl_2$ and primer concentration at 8 pM.

Optimization was refined by varying probe concentration (100 nM, 200 nM, 300 nM) at a concentration of 4 mM $MgCl_2$ and 8pM primer concentration (Table 2).

Figure 1. Results of *Plasmodium* genus TaqMan assay optimization testing varying $MgCl_2$ concentration 3 mM, 4 mM, 5 mM, 6 mM using constant concentrations of primer at 8 pM and probe at 200 nM.

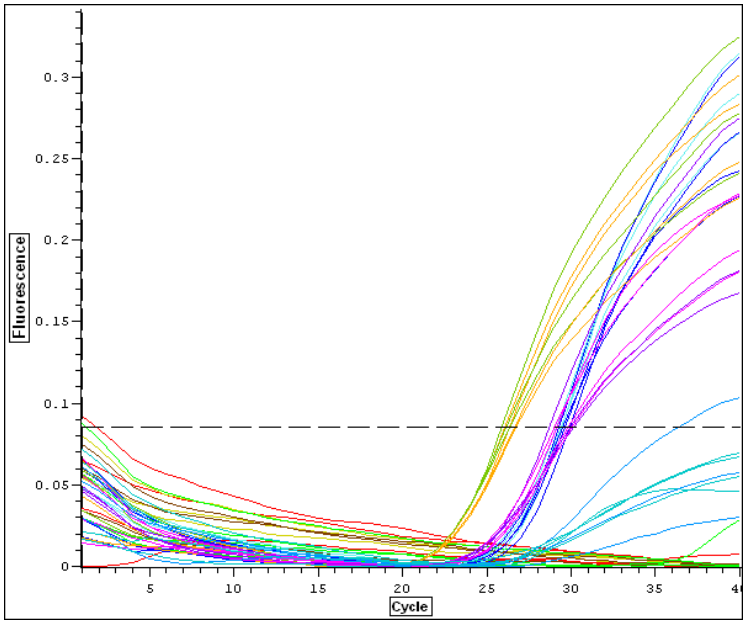


Table 1. Results of *Plasmodium* genus TaqMan assay optimization testing by varying $MgCl_2$ concentration 3 mM, 4 mM, 5, 6 mM using constant concentrations of primer at 8 pM and probe at 200 nM.

Description	C(t)
NTC MgCl 3 mM	N/A
NTC MgCl 4 mM	N/A
NTC MgCl 5 mM	N/A
NTC MgCl 6mM	N/A
NB MgCl 3 mM	N/A
NB MgCl 4 mM	N/A
NB MgCl 5 mM	N/A
NB MgCl 6 mM	N/A
P. vivax, MgCl 3 mM	30.27
P. vivax, MgCl 4 mM	29.77
P. viax, MgCl 5 mM	29.64
P. vivax, MgCl 6 mM	29.49
P. falciparum, MgCl 3 mM	26.75
P. falciparum, MgCl 4 mM	26.26
P. falciparum, MgCl 5 mM	26.48
P. falciparum, MgCl 6 mM	26.59
P. ovale, MgCl 3 mM	29.58
P. ovale, MgCl 4 mM	29.11
P. ovale, MgCl 5 mM	29.53
P. ovale, MgCl 6 mM	30.86
P. malariae, MgCl 3 mM	N/A
P. malariae, MgCl 4 mM	37.97
P. malariae, MgCl 5 mM	N/A
P. malariae, MgCl 6 mM	N/A

NTC = non-template control, NB = normal human blood

Table 2. Results of *Plasmodium* genus TaqMan assay optimization by varying probe concentrations of 100 nM, 200 nM, 300 nM using constant concentrations of 4 mM $MgCl_2$ and 8pM primer.

Description	C(t)
NTC ,100 nM probe	N/A
NTC ,200 nM probe	N/A
NTC ,300 nM probe	N/A
NB ,100 nM probe	N/A
NB ,200 nM probe	N/A
NB ,300 nM probe	N/A
P. vivax,100 nM probe	29.92
P. vivax,200 nM probe	29.78
P. vivax, 300 nM probe	30.01
P. falciparum,100nM probe	27.28
P. falciparum,200nM probe	26.86
P. falciparum,300 nM probe	27.14
P. ovale,100 nM probe	30.73
P. ovale,200nM probe	30.64
P. ovale,300nM probe	30.43
P. malariae,100 nM probe	N/A
P. malariae,200 nM probe	N/A
P. malariae,300 nM probe	N/A

NTC = non-template control, NB = normal human blood

Linearity and Limit of Detection.

The linearity of the assay was assessed in order to determine the amplification efficacy and efficiency of the PCR. These data were used to estimate LoD. The estimated value served as the starting point for further evaluation of LoD by replicate sample test. The correlation coefficient (R^2) of standard DNA concentrations was used to establish linearity. The slope was used to calculate amplification efficacy and efficiency using the formulas, Efficacy = $-1 + 10^{(-1/slope)}$ and Efficiency = $10^{(-1/slope)}$. The LoD was estimated using a standard curve produced by plotting Ct values versus the logarithm of eight serial dilutions of template, 10e-1 to 10e6 genomic copies, genomic equivalents (GE), per reaction volume. The Ct values of each log DNA concentration were measured in six replicates. Least-squares regression analysis plotted Ct as a function of DNA concentration. Instrument software automatically calculated "best-fit" of the regression and a standard curve was established, the linear relationship between Δ PCR cycle number and Δ DNA concentration. The R^2 value was automatically adjusted near or at unity by the RAPID software. The LoD was estimated as the template concentration at the lowest Ct value above background.

The LoD of the *Plasmodium* genus was established at 10 GE using *P. vivax* plasmid and *P. falciparum* plasmid (Figure 1 and Table 2). The optimized formulation was used; 4mM $MgCl_2$, 8pmole primers, and 200 nM probe.

Figure 2. Results of *Plasmodium* genus TaqMan assay LoD testing using the optimized formulation

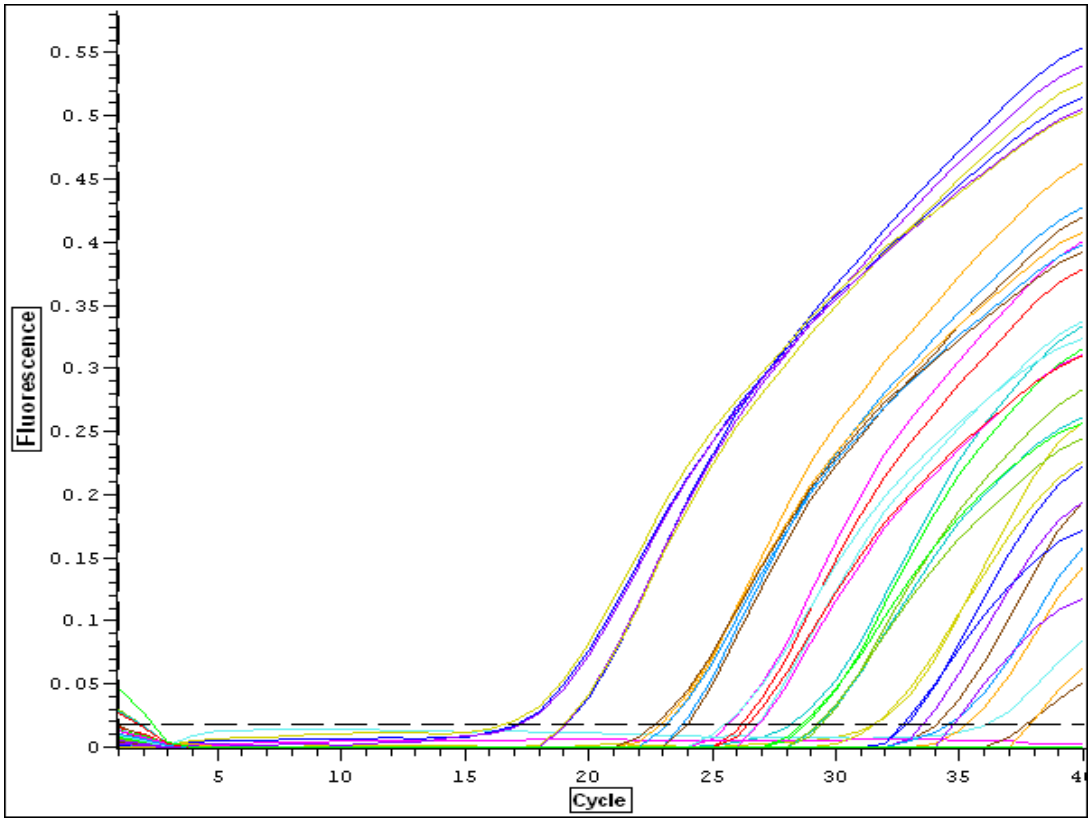


Table 3. Results of *Plasmodium* genus TaqMan assay LoD testing using the optimized formulation

Description (Copy number/ reaction)	C(t)
PV,10e6	19.11
PV,10e6	19.07
PV,10e6	19.03
PF,10e6	17.09
PF,10e6	17.15
PF,10e6	16.57
PV,10e5	23.14
PV,10e5	23.76
PV,10e5	23.95
PF,10e5	22.86
PF,10e5	23.16
PF,10e5	22.64
PV,10e4	25.63
PV,10e4	26.19
PV,10e4	26.83
PF,10e4	26.87
PF,10e4	26.43
PF,10e4	25.49
PV,10e3	28.15
PV,10e3	28.77
PV,10e3	29.38
PF,10e3	29.32
PF,10e3	28.61
PF,10e3	29.25
PV,10e2	31.44
PV,10e2	32.83
PV,10e2	33.47
PF,10e2	31.52
PF,10e2	32.7
PF,10e2	34.75
PV,10	34.05
PV,10	35.25
PV,10	34.57
PF,10	37.68
PF,10	37.79
PF,10	N/A
PV,1	36.06

PV,1	N/A
PV,1	N/A
PF,1	N/A
PF,1	N/A
PF,1	N/A
NTC	N/A
PV,0.1	N/A
PV,0.1	N/A
PV,0.1	N/A
PF,0.1	N/A
PF,0.1	N/A
PF,0.1	N/A
NTC	N/A

PV = *P. vivax* plasmid, PF= *P. falciparum* plasmid

Sensitivity Testing

The *Plasmodium* genus Taqman assay was 100% (17/17) concordant in testing using diverse panel of *Plasmodium* species DNA extracted from patient bloods (Figure 3, Table 4).

Figure 3. Results of the *Plasmodium* genus TaqMan sensitivity testing using *Plasmodium* plasmid control DNA

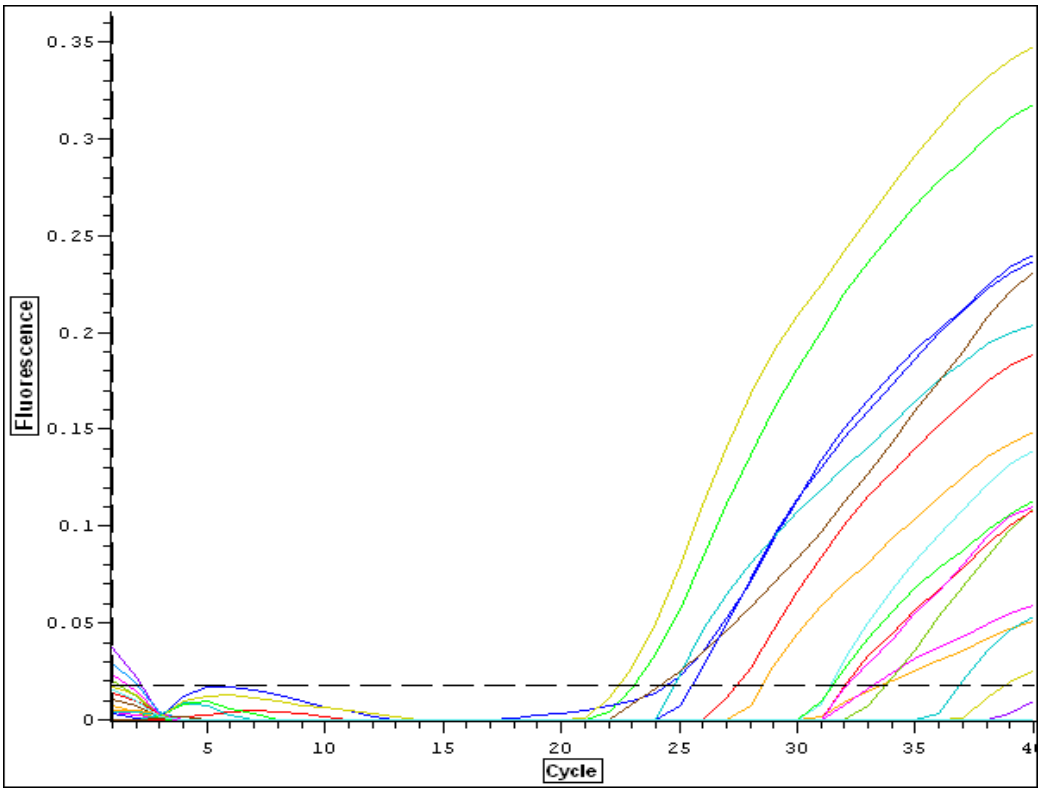


Table 4. Results of *Plasmodium* genus TaqMan assay sensitivity testing

Description	C(t)
PF 424	23.09
PV 1882	24.4
PM 21	33.55
*P.knowlesi**	33.18
PV 75	27.4
*P.berghei**	24.75
PV 76	31.44
PV 135	31.49
PF 447	25.52
PF 12	22.42
PV 63	38.82
PV 80	33.72
PV 137	32.02
PV 117	28.52
PF 5	36.85
NTC	N/A
PV 69	24.17
PV 81	N/A
PV 138	N/A
PV 3157	32.12
NTC	N/A

Note: PF = *P. falciparum*, PV = *P. vivax*, PM = *P. malariae;*
(*) = DNA samples were extracted from sporozoites (prototype *Plasmodium* species)

Sensitivity and Cross-reactivity Testing

The *Plasmodium* genus TaqMan assay was 100% (5/5) sensitive and 100% (13/13) specific in testing with a diverse panel of human malaria *Plasmodium* species and non-malaria pathogens (Figure 4, Table 5). No cross-reaction was observed using human blood samples.

Figure 4. Results of the *Plasmodium* genus TaqMan sensitivity and cross-reactivity testing

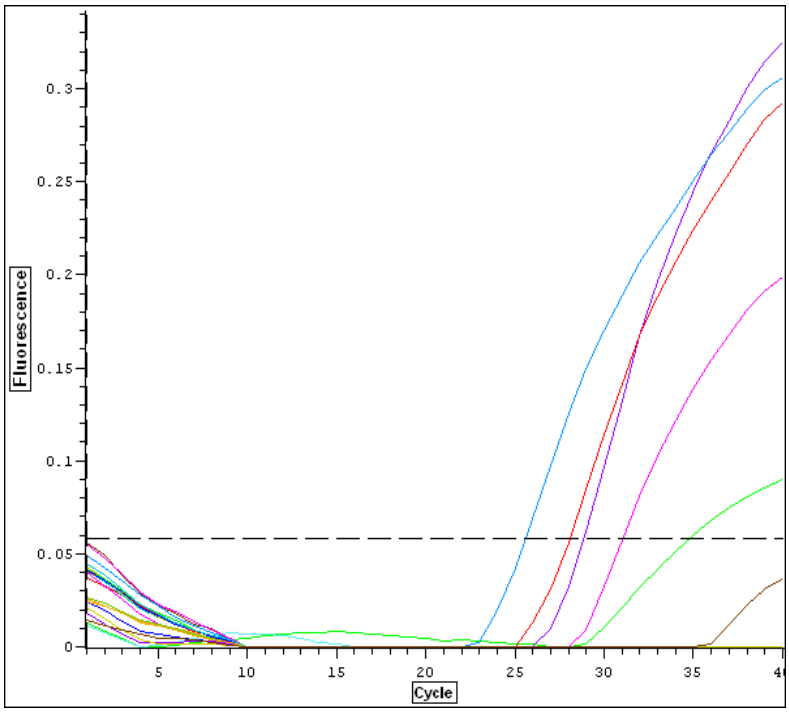


Table 5. Results of the *Plasmodium* genus TaqMan assay sensitivity and cross-reactivity testing

Description	C(t)
NTC 8 pmole	N/A
Normal blood 8 pmole	N/A
PV 8 pmole	28.87
PF 8 pmole	25.59
PO 8 pmole	28.06
PM 8 pmole	34.77
E.coli 8 pmole	N/A
P.aeruginosa 8 pmole	N/A
S.typhimurium 8 pmole	N/A
K.pneumoniae 8 pmole	N/A
E.aerogenes 8 pmole	N/A
S.aureus 8 pmole	N/A
S.flexneri 8 pmole	N/A
S.sonnei 8 pmole	N/A
S.pyogenes 8 pmole	N/A
Bartonella 8 pmole	N/A
P.knowlesi 8 pmole	31.03
Rickettsia 8 pmole	N/A
Orientia 8 pmole	N/A
Leptospira 8 pmole	N/A

Note: PF = *P. falciparum*, PV = *P. vivax*, PM = *P. malariae*, PO = *P. ovale*

Conclusion

A highly sensitive and specific *Plasmodium genus* TaqMan PCR assay was developed using real-time PCR instrumentation (MiniOpticon Real-Time PCR System, Bio-Rad, Hercules, CA). This assay is applicable for use with real-time PCR instrumentation to include RAPID/JBAIDS.

The LoD of the *Plasmodium* genus assay was established at 10 genome equivalents using *P. vivax* plasmid and *P. falciparum* plasmid. The assay was 100% (17/17) concordant in testing using a diverse panel of *Plasmodium* species and strains prepared as plasmid control DNA. The assay was 100% (5/5) sensitive and 100% (13/13) specific in testing with a diverse panel of human malaria *Plasmodium* species and non-malaria pathogens. No cross-reaction occurred with human blood samples. Transfer to the RAPID/JBAIDS presents no technical challenges.

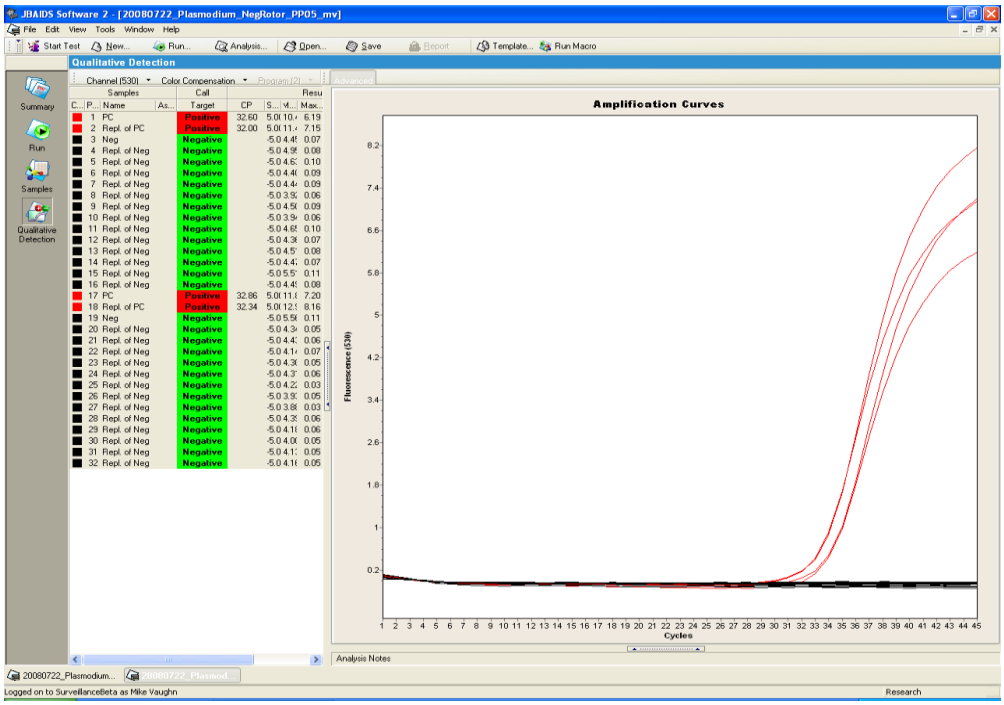
The *Plasmodium genus* assay is sensitive, specific, and provides rapid detection from infected mosquitoes and malaria patient blood. Operational applications are FHP disease surveillance and ultimately molecular-based diagnostics. A unique 18S rRNA gene sequence conserved in *Plasmodium* species was targeted for the design of oligonucleotide primers and probe. Assay chemistry and formulation allows use on diverse real-time PCR analytic platforms. The *Plasmodium genus* assay is a likely candidate for transfer to the DoD NGDS.

Malaria causative agent detection capability was accomplished during March through May 2012. A highly sensitive and specific *Plasmodium* genus TaqMan PCR assay was developed and successfully tested.

Appendix A

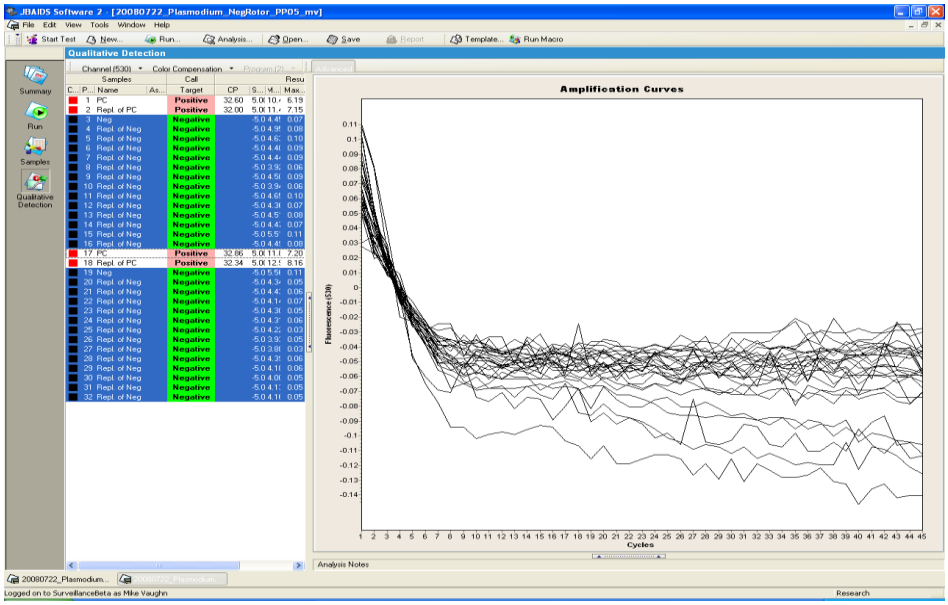
Idaho Technology Inc. Freeze-dried Reagent QC Results

20080722_Plasmodium_NegRotor#1_PP05_mv

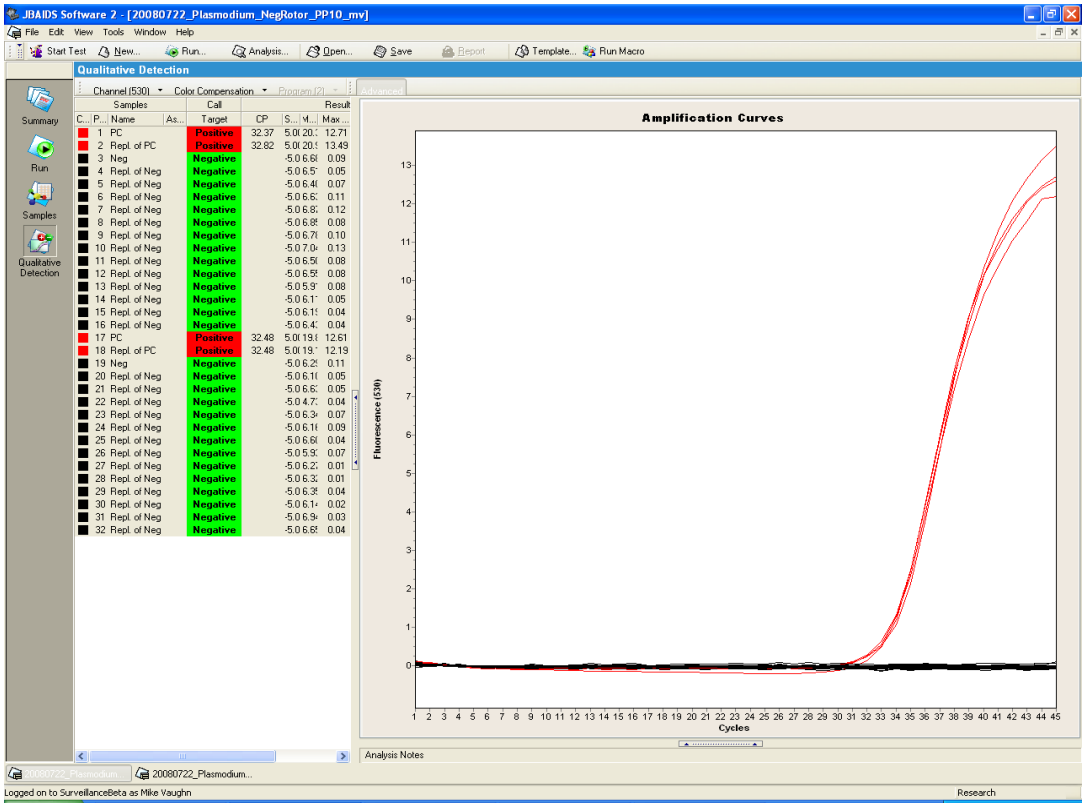


SUMMARY:

		Crossing Point			Max Fluorescence			Detector Calls		
Cond#:	Condition:	AVE	ST DEV	%CV	AVE	ST DEV	%CV	P	N	U
1	Repl. of PC	32.45	0.37	1.13	7.17	0.80	11.16	4	0	0
2	Repl. of Neg	-	-	-	0.07	0.02	30.99	0	28	0

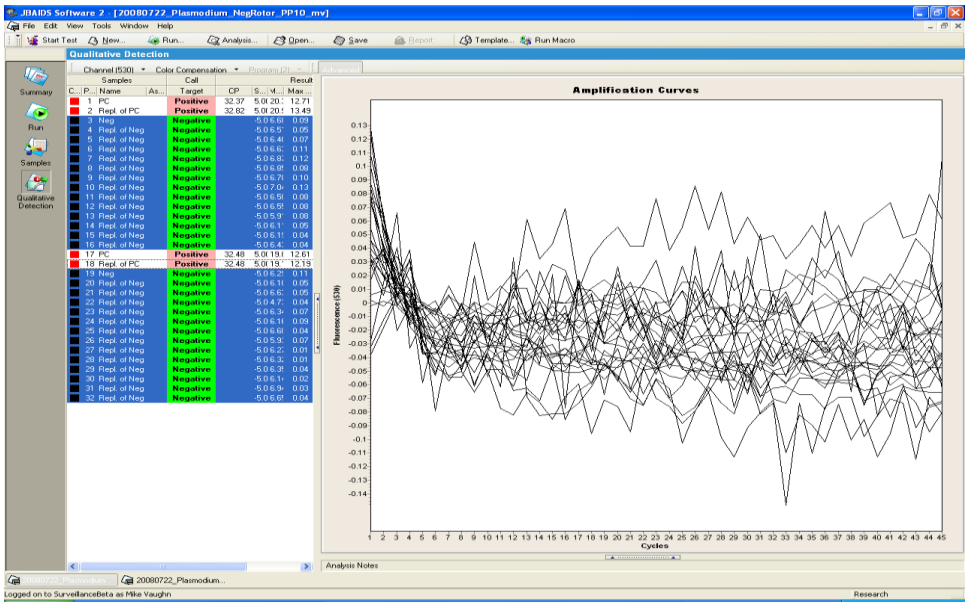


20080722_Plasmodium_NegRotor#2_PP10_mv

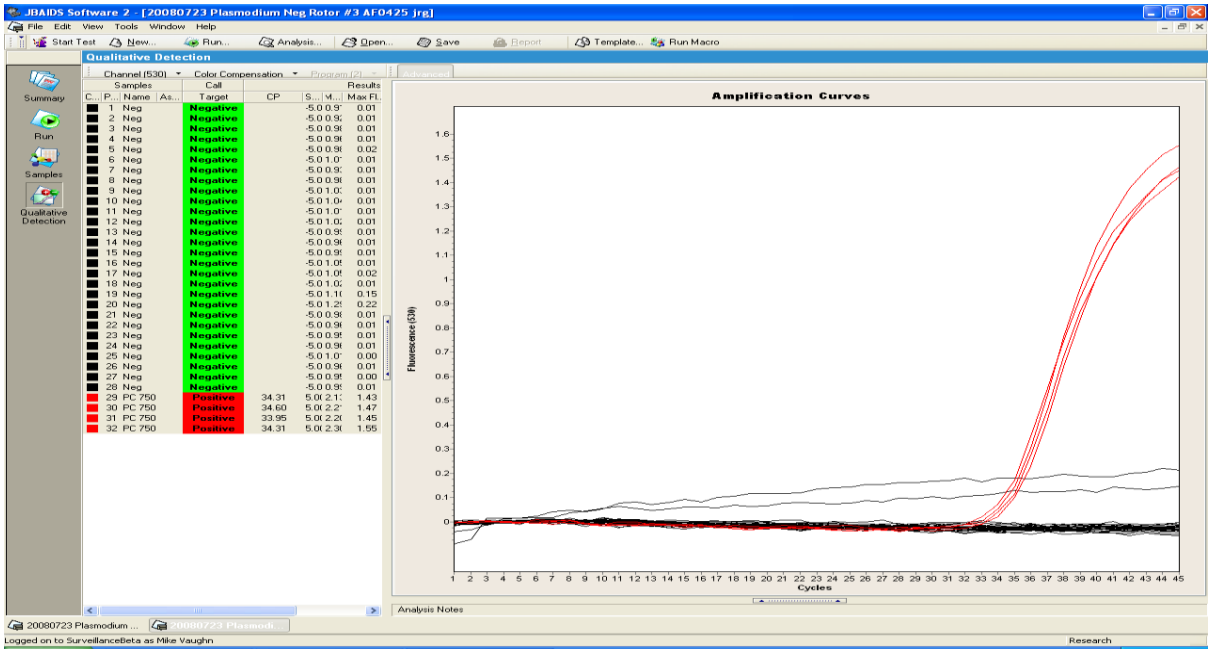


SUMMARY:

		Crossing Point			Max Fluorescence			Detector Calls		
Cond#:	Condition:	AVE	ST DEV	%CV	AVE	ST DEV	%CV	P	N	U
1	Repl. of PC	32.54	0.20	0.60	12.75	0.54	4.26	4	0	0
2	Repl. of Neg	-	-	-	0.06	0.03	51.00	0	28	0

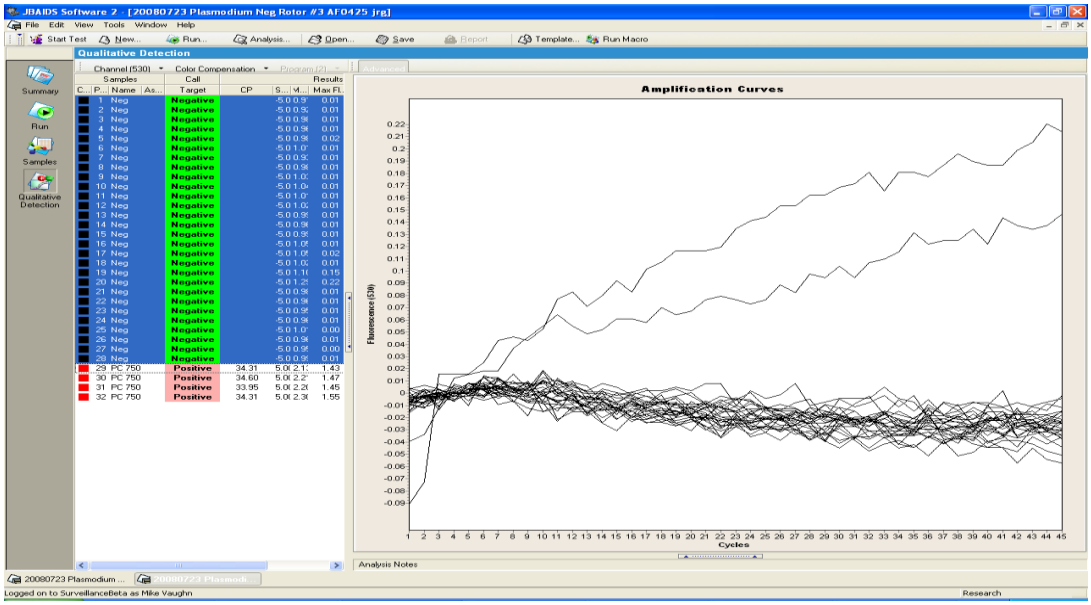


20080723 Plasmodium Neg Rotor #3 AF0425 jrg

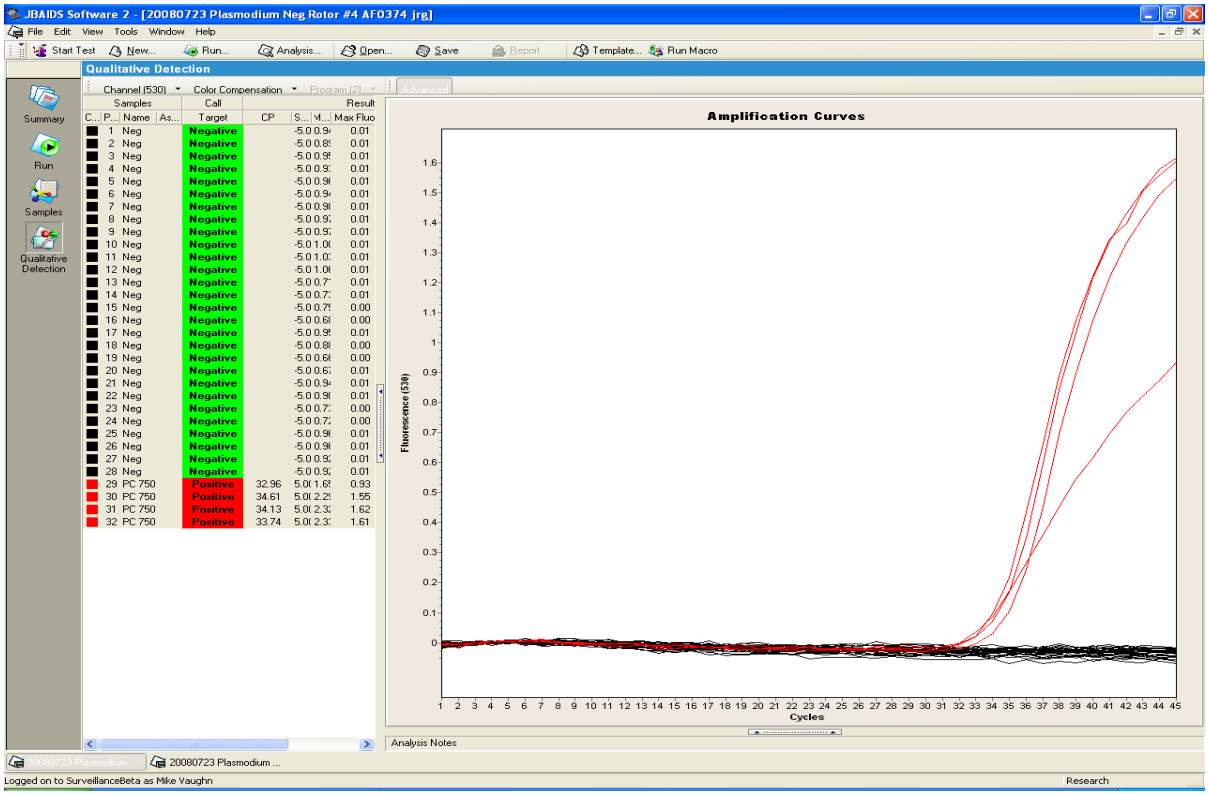


SUMMARY:

		Crossing Point			Max Fluorescence			Detector Calls		
Cond#:	Condition:	AVE	ST DEV	%CV	AVE	ST DEV	%CV	P	N	U
2	Neg	-	-	-	0.02	0.05	210.25	0	28	0
3	PC 750 fg	34.29	0.27	0.78	1.47	0.06	3.83	4	0	0


JBAIDS Software 2 - [20080723 Plasmodium Neg Rotor #3 AF0425 jrg]
File Edit View Tools Window Help
Start Test New... Run... Analysis... Open... Save Report Template... Run Macro
Qualitative Detection
Summary
Run
Samples
Qualitative Detection
Channel (530) Color Compensation
Samples Call Results
C... P... Name As... Target CP S... M... Max Fl...
1 Neg Negative 0.01
2 Neg Negative 0.01
3 Neg Negative 0.01
4 Neg Negative 0.01
5 Neg Negative 0.02
6 Neg Negative 0.01
7 Neg Negative 0.01
8 Neg Negative 0.01
9 Neg Negative 0.01
10 Neg Negative 0.01
11 Neg Negative 0.01
12 Neg Negative 0.01
13 Neg Negative 0.01
14 Neg Negative 0.01
15 Neg Negative 0.01
16 Neg Negative 0.01
17 Neg Negative 0.02
18 Neg Negative 0.01
19 Neg Negative 0.15
20 Neg Negative 0.22
21 Neg Negative 0.01
22 Neg Negative 0.01
23 Neg Negative 0.01
24 Neg Negative 0.01
25 Neg Negative 0.00
26 Neg Negative 0.01
27 Neg Negative 0.00
28 Neg Negative 0.01
29 PC 750 Positive 34.31 5.0 2.1 1.43
30 PC 750 Positive 34.60 5.0 2.2 1.47
31 PC 750 Positive 33.95 5.0 2.2 1.45
32 PC 750 Positive 34.31 5.0 2.3 1.55
Amplification Curves
Fluorescence(530)
Cycles
Analysis Notes
20080723 Plasmodium ...
Logged on to SurveillanceBeta as Mike Vaughn
Research

20080723 Plasmodium Neg Rotor #4 AF0374 jrg



SUMMARY:

		Crossing Point			Max Fluorescence			Detector Calls		
Cond#:	Condition:	AVE	ST DEV	%CV	AVE	ST DEV	%CV	P	N	U
2	Neg	-	-	-	0.01	0.00	37.83	0	28	0
3	PC 750 fg	33.86	0.70	2.06	1.43	0.33	23.11	4	0	0